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TITLE:

A Revised Method for Inducing Secondary Lymphedema in the Hindlimb of Mice

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SUMMARY:

This animal model enables researchers to induce statistically significant secondary lymphedema in the hindlimb of mice, lasting at least 8 weeks. The model can be used to study the pathophysiology of lymphedema and to investigate novel treatment options.

ABSTRACT:

Animal models are of paramount importance in the research of lymphedema in order to understand the pathophysiology of the disease but also to explore potential treatment options. This mouse model allows researchers to induce significant lymphedema lasting at least 8 weeks. Lymphedema is induced using a combination of fractioned radiotherapy and surgical ablation of lymphatics. This model requires that the mice get a dose of 10 Gray (Gy) radiation before and after surgery. The surgical part of the model involves ligation of three lymph vessels and extraction of two lymph nodes from the mouse hindlimb. Having access to microsurgical tools and a microscope is essential, due to the small anatomical structures of mice. The advantage of this model is that it results in statistically significant lymphedema, which provides a good basis for evaluating different treatment options. It is also a great and easily available option for microsurgical training. The limitation of this model is that the procedure can be time consuming, especially if not practiced in advance. The model results in objectively quantifiable lymphedema in mice, without causing severe morbidity and has been tested in three separate projects.

INTRODUCTION:

Lymphedema is characterized by an accumulation of lymph fluid that leads to localized tissue swelling, which mainly occurs due to impaired or disrupted flow of lymph fluid in the lymphatic

vessels[1]. The lymph flow can be impaired or disrupted by infection, obstruction, injury or congenital defects in the lymphatic system[2]. These etiologies result in accumulation of lymphatic fluid, which leads to a chronic state of inflammation, resulting in subsequent fibrosis, as well as deposition of adipose tissue[3]. Lymphedema can be categorized as primary or secondary lymphedema. Primary lymphedema is caused by developmental abnormalities or genetic mutation[2,4]. Secondary lymphedema occurs due to underlying systemic disease, surgery or trauma[2,4]. Secondary lymphedema is the most common form of lymphedema in the world[2]. In developed countries, the most common cause of secondary lymphedema is oncological therapy such as adjuvant radiotherapy and lymph node dissection[5]. Lymphedema is most frequent among breast cancer patients, but can also develop in patients with gynecologic, melanoma, genitourinary or neck cancer[6]. It has been suggested that out of all women diagnosed with breast cancer, 21% will develop lymphedema[7].

Lymphedema can be stressful to the patient both physically and psychologically. Patients with lymphedema have an increased risk of infection[5,8,9], poor quality of life and can develop social anxiety and symptoms of depression[10]. The complications of chronic lymphedema lead to high cost of care and an increased disease burden[9,11]. Findings have also suggested that lymphedema might be associated with increased risk of death after breast cancer treatment[12]. Conservative management such as compression of the affected area, manual lymph drainage and general skincare remain the first line approach. There is currently no curative treatment[6]. Although progress has been made in the field of surgical and medical therapy, there is still room for improvement. More research, providing insight in the pathophysiology and progression of the disease, is needed to enable clinicians to provide better treatment options for the patients[5].

Animal models are being used in preclinical research to understand the pathophysiology of diseases and develop potential treatment options. Several different lymphedema animal models have been established in canines[13,14], rabbits[15], sheep[16], pigs[17,18] and rodents[19-24]. The rodent model seems to be the most cost-effective model, when investigating the reconstruction of lymphatic function, due to rodents being easily accessible and relatively low-priced[25]. The majority of the mice models have focused on inducing lymphedema in the tail of the mice[21-23]. The tail model is very reliable but the exact surgical technique for inducing lymphedema varies significantly in previous published material. This results in fluctuations in duration and robustness of the developed lymphedema presented in known litterature[25]. Different techniques are also being used for inducing lymphedema in the hindlimb model and they also yield varying results, but the hindlimb model might be easier to understand from a translational perspective. Previous lymphedema models have been hampered by spontaneous lymphedema resolution and therefore a reproducible and permanent lymphedema model is needed[25]. Researchers have previously tried to increase the dose of radiation, to prevent the spontaneous lymphedema resolution, but this has often led to subsequent severe morbidity[25].

This model results in statistically significant lymphedema, without causing severe morbidity, by combining microsurgery with radiation. The model has been revised from a previous surgical model by adding a dose of irradiation that induces lymphedema, without causing severe morbidity[26]. It also offers a great opportunity for microsurgical training. Having access to

microsurgical equipment and a microscope is necessary, due to the small anatomical structures of the mice. The surgical procedure can be performed when the user has been taught basic microsurgical techniques, such as suturing with microsurgical instruments. The operators that performed this procedure all watched tutorial videos by Acland on the preconditions of microsurgical skills (1981) and basic microsuture technique (1985). We recommend practicing the surgical procedure 8−10 times before using it in research. Practicing the procedure ensures that fewer mistakes are made and that the procedure can be performed more efficiently. When mastered, the surgical procedure can be performed in 45 minutes.

PROTOCOL:

Animals were housed in the University of Southern Denmark Animal Care Facility as per institutional guidelines. All procedures involving animal subjects have been approved by The Animal Experiments Inspectorate, Ministry of Environment and Food of Denmark.

1. Pre-surgery irradiation

NOTE: Pre-surgery irradiation takes place 7 days before surgery.

1.1. Induce anesthesia.

1.1.1. Place the mouse in an induction box and set the vaporizer to 3% isoflurane with an oxygen flow rate of 0.8−1.2 L/min to induce inhalation anesthesia.

NOTE: Alternatively, injectable anesthetics can be used but for the short duration of the irradiation inducing inhalation anesthesia was sufficient. For obtaining the results presented in this article, 9-week old female C57BL6 mice were used.

1.1.2. Make sure the mouse is fully anesthetized by tail or paw pinch test.

1.2. Position the mouse for irradiation.

1.2.1. If fully sedated, move the mouse from the induction box and place it under the source of radiation in supine position and gently fixate the hind limbs with tape.

NOTE: The mouse will remain sedated for the short duration of the radiation.

1.2.2. Place a 1.5 mm thick lead pad to ensure that only the area that undergoes surgery (i.e., the circular area with a diameter of 25 mm around the knee) gets irradiated.

1.3. Administer a dose of 10 Gy radiation at a dose rate of 5.11 Gy/min (100 kVp, 10 mA).

CAUTION: Safety precautions must be taken when working with radiation. During this

experiment, all irradiation was performed in a radiation insulated room, and the source of radiation was only turned on when all personnel had left and sealed the room.

1.4. Place the mouse back in its cage.

2. Equipment setup

NOTE: Surgery should be performed in a room dedicated to surgical procedures. The operative surface must be sterile.

2.1. Thoroughly clean all operative surfaces with 70% ethanol. Wear hairnet and coveralls. Use sterile surgical instruments and sterile gloves.

2.2. Prepare anesthesia.

2.2.1. Draw up 1 mL of fentanyl (0.315 mg/mL), 1 mL of midazolam (5 mg/mL), and 2 mL of sterile water. Use different syringes and needles for the different components.

2.2.2. Mix fentanyl and sterile water by slowly emptying the syringes into a sterile glass tube. When mixed, add midazolam to complete the working solution.

2.3. Prepare analgesia.

2.3.1. Draw up 0.2 mL of buprenorphine (0.3 mg/mL) and 2 mL of saline.

2.3.2. Mix the volumes by slowly emptying the syringes into a sterile glass tube to complete the working solution.

2.4. Turn on the microscope and make sure that the lighting is sufficient, and that the microscope is well adjusted for the operator's eyes.

NOTE: All surgical procedures should be performed under an operating microscope. A magnification range from 4x−25x is sufficient.

3. Preparation

3.1. Weigh the mouse pre-surgery by placing the mouse in an empty container on a cleared scale.

3.2. Administer anesthetic.

3.2.1. Draw up 0.1 mL of anesthetic per 10 g of mouse bodyweight. Inject the anesthetic subcutaneously as a bolus injection.

3.2.2. Let the mouse rest in a cage with plenty bedding and shelter for approximately 10 min until

fully sedated. Examine the anesthetic depth by assessing muscle relaxation and perform paw or tail pinch test.

3.3. When fully sedated, shave the hind limb chosen for the procedure using electrical clippers. Make sure to wipe of excess hair.

3.4. Turn on the heating device, such as a heating pad and cover it with a surgical cloth.

3.5. Set the flow of oxygen to 0.8 L/min and connect it with a nosecone. Use 100% oxygen.

NOTE: The nosecone is only for oxygen delivery and not anesthesia.

3.6. Apply ophthalmic ointment and inject 0.5 mL of saline subcutaneously, preferably in the scruff of the mouse, to prevent hypovolemia during surgery.

3.7. Position the mouse for surgery.

3.7.1. Place the mouse on the surgical cloth in supine position. Place the nosecone over the snout.

3.7.2. Fixate the end of the hindlimbs gently with tape to prevent the mouse from shifting during surgery.

3.7.3. Sterilize the skin using alcohol/chlorhexidine or alcohol/povidone iodine.

4. Surgery

NOTE: In this example, the left hind limb (when the mouse is viewed in supine position), has been chosen for the procedure.

4.1. Make a circular incision.

4.1.1. Lift the skin with smooth forceps and clip a small opening approximately 5 mm proximal to the popliteal fossa.

4.1.2. Slide sharp scissors into the opening and clip towards the knee so that the incision ends just above the knee. Make sure not to puncture the underlying vessels by lifting the skin with forceps while clipping.

4.1.3. Move the mouse to prone position and continue to clip from the knee towards the popliteal fossa until the circumferential incision is complete.

4.2. Dissect the skin below the knee.

4.2.1. Gently blunt dissect the area below the knee to a couple of millimeters above the ankle, by slowly opening and closing the microscissors while lifting the skin with forceps.

4.2.2. Carefully snip remaining visible adhesions using microscissors. Use sterile saline regularly to keep the tissue moist during the whole procedure.

4.3. Dissect the skin at the proximal rim of the circumferential incision so that it can be retracted with an elastic retractor.

NOTE: The retractor allows the operator a better view of the proximal lymph vessel and prevents the proximal rim from shifting during surgery.

4.4. While still in prone position, rotate the hindlimb gently and fixate it with tape, so that the ischiatic vein is visible from the most proximal point of the exposed area to the most distal point.

4.5. Inject approximately 0.01 mL of Patent Blue V subcutaneously between the second and third toe using a 0.5 mL syringe with a 30 G needle. Gently press the paw a couple of times to distribute the Patent Blue V. Visualize the lymph vessels and lymph node through the microscope as the Patent Blue V fills the lymph vessels.

NOTE: If the blue color of the lymph vessels fades during the procedure, gently massage the paw to promote uptake, rather than inject more Patent Blue V. Excess use of Patent Blue V may lead to leakage and coloring of the tissue surrounding the lymph vessels which may compromise the procedure.

4.6. Locate the important structures: the popliteal lymph node (PLN), the two lymph vessels distal to the lymph node (DLV1 and DLV2), and the one lymph vessel proximal to the lymph node (PLV).

NOTE: All the lymph vessels can be found adjacent to the ischiatic vein. The proximal lymph vessel is usually found medial to the vein, the two distal lymph vessels are found medial and lateral to the vein. The abbreviations of the structures are used in the accompanying video.

4.7. Magnify to clearly visualize the PLV and ligate it with a 10-0 nylon suture using micro-needle holder and microforceps. Press the paw a couple of times to ensure that no Patent Blue V passes proximal to the suture.

NOTE: Trimming the fat surrounding the lymph vessel may be necessary.

4.8. Repeat step 4.7 to ligate the two distal lymph vessels. Press the paw several times to ensure that no Patent Blue V passes proximal to the ligature. If the lymph vessels lie to close to the ischiatic vein, try dissecting even further distally.

NOTE: In this example, it can be seen that one of the lymph vessels bursts due to the ligature hindering the lymph flow. The lymph vessels will often split from the vein further down.

4.9. Remove the popliteal lymph node.

4.9.1. Locate the popliteal lymph node and clip a small hole with microscissors to access it and remove it with microforceps and microscissors.

NOTE: The lymph node has a smooth pearl-like surface in contrast to the surrounding fat tissue.

4.9.2. To test if the removed tissue is a lymph node, place it in a test tube filled with water.

NOTE: If the tissue is comprised of fat, the tissue will float. If the tissue is a lymph node, it will sink to the bottom.

4.10. Remove the inguinal fat pad and lymph node.

4.10.1. Before removing the inguinal fat pad, use a bipolar coagulator to cauterize the vessels running through the fat.

4.10.2. Resect the inguinal fat pad using microforceps and microscissors. Gently clip the cauterized vessels running through the fat. Then gently resect the fat tissue in the inguinal area.

NOTE: The lymph node located in the fat is rarely colored by Patent Blue V and can be hard to differentiate from the fat. Removing the fat pad in one piece is the best way to ensure the lymph node has been removed.

4.11. Rinse the leg thoroughly with sterile saline and confirm through the microscope that any small hairs and particles has been thoroughly removed from the surgical area to avoid wound contamination and infection. Make sure there is no active bleeding.

4.12. Suture the skin edges down to the muscle facia with a 6-0 nylon suture using forceps and needle holder, leaving a gap of 2−3 mm to constrain the superficial lymph flow.

NOTE: The accompanying video shows an example of finished sutures.

4.13. Administer analgesia. Draw up 0.1 mL of analgesia per 30 g of mouse bodyweight. Inject the analgesia subcutaneously as a bolus injection.

4.14. Weigh the mouse for post-surgery for comparison.

4.15. Place the mouse in a cage in a cabinet heated for recovery.

5. Postoperative care

5.1. Give the mice individual cages to recover after surgery with water and food ad libitum.

5.2. Administer a bolus subcutaneous dose of 0.02 mL of buprenorphine 3x daily for 3 days for analgesia.

5.3. Monitor the animal daily for appropriate wound healing, signs of pain and infection. If signs of infection are present, use antibiotic ointment.

6. Post-surgery irradiation

6.1. Three days after surgery, repeat the procedure for pre-surgery irradiation (steps 1.1–1.4).

REPRESENTATIVE RESULTS:

This procedure has previously been used in three separate experiments. All the experiments were made by different lead investigators who all are co-authors of this article. In all three experiments, great care was taken to adhere to the same procedure as described in this protocol. In all three experiments, secondary lymphedema was induced in one hindlimb while the other hindlimb served as a control. Volumes of the hindlimbs were the primary outcome in all three experiments. **Figure 1** illustrates the study design.

All mice underwent micro-computed tomography (μCT) scans in the weeks following surgery to measure the volume of the hindlimbs. The μCT scans were performed on a multimodality pre-clinical scanner (**Table of Materials**) and the volume of the hindlimbs was measured via the region-of-interest (ROI) function in the associated software as previously described[26]. The distal tibiofibular joint was located in three-dimensional (3D) axonal images using a method previously described[27]. The ROI started at the distal tibiofibular joint and included all tissue distal to that point. The Hounsfield range for the analysis was set to -500 to 4000.

All data were analyzed using statistical software (**Table of Materials**). Sidak's multiple comparison test was used to compare the volume of the induced lymphedema hindlimb, with the control hindlimb. A significant difference between the control hindlimb and lymphedema hindlimb is defined as a P-value <0.05.

[Place **Figure 1** here]

Experiment 1[26] included 32 mice distributed into groups of four. One of the objectives was to study several different doses of radiation and find the most preferable dose, for inducing lasting lymphedema without causing severe morbidity. The group that was given two doses of 10 Gy irradiation included four mice. **Figure 2** shows that a consistent state of lymphedema was achieved in all 8 weeks. **Table 1** shows that there was a significant difference in volume between the lymphedema hindlimb and control hindlimb in weeks 1, 7, and 8. While a consistent state of induced lymphedema was achieved, there was not a statistically significant difference between the hindlimbs during all 8 weeks. This outcome differs from the two other experiments and could

be explained due to the relatively smaller sample size of four mice. Increasing the number of measurements would increase the power of the study and hereby the probability of detecting a difference if a difference exists[28].

[Place **Figure 2** here]

[Place **Table 1** here]

Experiment 2 included 45 mice. 15 mice served as controls and were given saline injections. The controls are used as representative results as we assume that the saline injections had no effect on the volume of induced lymphedema. **Figure 3** shows that the lymphedema was less stable than in experiment 1. Additionally, the volume of the control hindlimbs increased during the 8 weeks. This decreases the relative difference presented in **Table 2**. It has been speculated that the mice use their non-operated hindlimb more, in the weeks following surgery, and that this leads to hypertrophy and increase in limb volume of the non-operated hindlimb. Most importantly, **Table 3** shows that there is statistically significant difference between the lymphedema hindlimb and the control hindlimb during all 8 weeks after surgery. The higher number of mice proves that this procedure can induce statistically significant lymphedema for at least 8 weeks.

[Place **Figure 3** here]

[Place **Table 2** here]

[Place **Table 3** here]

Experiment 3 included 36 mice. 12 mice served as controls and were given saline injections. The controls are used as representative outcome as we assume that the saline injections had no effect on the volume of induced lymphedema. In this experiment the hindlimb volume of the mice were measured for 6 weeks instead of 8. The experiment only lasted 6 weeks due to logistical difficulties when the experiment was performed. **Figure 4** shows a more consistent lymphedema than experiment 2. **Table 4** shows that there is statistically significant lymphedema in the 6 weeks after surgery.

[Place **Figure 4** here]

[Place **Table 4** here]

Figure 5 and **Table 5** shows the mean hindlimb volume of all three experiments combined. **Table 5** shows that the use of this procedure results in statistically significant lymphedema lasting at least 8 weeks. Data from the first 6 weeks, are the combined measurements of 31 mice from experiments 1, 2 and 3. In week 7−8 we only had data from experiments 1 and 2 resulting in combined measurements from 19 mice.

[Place **Figure 5** here]

[Place **Table 5** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Study design and time points for outcome measurements.

Figure 2: Mean hindlimb volume: Experiment 1. Measurements of 4 mice from the group that was given two doses of 10 Gy irradiation are included in this figure. This graph shows the mean hindlimb volumes in mm^3 in the 8 weeks after surgery. All mice received a dose of 10 Gy irradiation pre- and post-surgery. The error bars represent the standard deviation (SD).

Figure 3: Mean hindlimb volume: Experiment 2. Measurements of 15 mice from the control group are included in this figure. This graph shows the mean hindlimb volumes in mm^3 in the 8 weeks after surgery. All mice received a dose of 10 Gy irradiation pre- and post-surgery. The error bars represent SD.

Figure 4: Mean hindlimb volume: Experiment 3. Measurements of 12 mice from the control group are included in this figure. This graph shows the mean hindlimb volumes in mm^3 in the 6 weeks after surgery. All mice received a dose of 10 Gy irradiation pre- and post-surgery. The error bars represent SD.

Figure 5: Combined mean hindlimb volume: Experiment 1, 2 and 3. Thirty-one mice included in the first 6 weeks after surgery and 19 mice included in the following 2 weeks. This graph shows the mean hindlimb volumes in mm^3 in the 8 weeks after surgery. All mice received a dose of 10 Gy irradiation pre- and post-surgery. The error bars represent SD.

Table 1: Sidak's multiple comparisons test: Experiment 1. This table shows the statistical comparison between the mean volumes of induced lymphedema hindlimbs and control hindlimbs during the 8 weeks after surgery. All mice received a dose of 10 Gy irradiation pre- and post-surgery. Values are presented as: mean ± SD in mm^3. P-value < 0.05 is considered as a significant difference between the control hindlimb and lymphedema hindlimb. n (number of observations) = 4.

Table 2: Absolute and relative difference. This table shows the absolute difference in volume between lymphedema- and control hindlimbs ± SD in mm^3 and the relative difference ± SD in percent.

Table 3: Sidak's multiple comparisons test: Experiment 2. This table shows the statistical comparison between the mean volumes of induced lymphedema hindlimbs and control hindlimbs in the 8 weeks after surgery. All mice received a dose of 10 Gy irradiation pre- and post-surgery. Values are presented as: mean ± SD in mm^3. P-value < 0.05 is considered as a

significant difference between the control hindlimb and lymphedema hindlimb. n (number of observations) = 15.

Table 4: Sidak's multiple comparisons test: Experiment 3. This table shows the statistical comparison between the mean volumes of induced lymphedema hindlimbs and control hindlimbs in the 6 weeks after surgery. All mice received a dose of 10 Gy irradiation pre- and post-surgery. Values are presented as: mean ± SD in mm^3. P-value < 0.05 is considered as a significant difference between the control hindlimb and lymphedema hindlimb. n (number of observations) = 12.

Table 5: Sidak's multiple comparisons test: Experiment 1, 2 and 3 combined. This table shows the statistical comparison between the mean volumes of induced lymphedema hindlimbs and control hindlimbs of 31 mice in the first 6 weeks after surgery and 19 mice in the following 2 weeks. All mice received a dose of 10 Gy irradiation pre- and post-surgery. Values are presented as: mean ± SD in mm^3. P-value < 0.05 is considered as a significant difference between the control hindlimb and lymphedema hindlimb. n (number of observations) = 31.

DISCUSSION:

There are a few critical steps in this protocol. Firstly, it is important that the researchers take safety precautions when working with radioactivity. Secondly, during the surgical part of this protocol, it is important to start the procedure once the mouse has been anesthetized and finish it without unnecessary breaks. This is important to avoid an excessively long surgical period for the animal and to prevent that the anesthesia loses effect during surgery. It is recommended to only administer one bolus injection of anesthetic and complete the surgical procedure in one sitting. It is also a critical step, not to administer too much Patent Blue V, as excess Patent Blue V will discolor the tissue surrounding the lymph vessels. If the surrounding tissue gets discolored it can be nearly impossible to visualize the lymph vessels and this compromises the procedure. Even if one does manage to visualize the lymph vessels, the discolored tissue will make it hard to assess whether the Patent Blue V passes proximal to the ligature or not. This is problematic because the operator must be sure that the placed ligatures are constricting the lymph flow, to ensure that the procedure will be successful. It is also important to leave a gap of 2−3 mm when closing the wound. As a temporary skin gap is often needed to mimic the human wound healing process[29].

The limitations of this method are that it is a time-consuming procedure that requires access to a microscope and previous microsurgical training. When performing the surgical part of this protocol, it is important to plan the time in-between the surgical procedures. A lot of time goes into waiting for the animal to be anesthetized, shaving the hindlimb and generally prepare for each surgical procedure. Therefore, it is recommended to prepare housing and anesthetic in advance. It is important to note that to be certain that chronic lymphedema has been induced, histopathology must be analyzed. We have not included histopathology in this article, which is a limitation. Without histopathology supporting the fact that histologic changes have happened to the lymph vessels the changes in volume in the hindlimbs can only be described as edema. The article that includes all data on the four mice from experiment 1[26] includes histopathology and

shows that there were significant changes to the histopathology using this technique. The article also includes lymphatic imaging. The same procedure was used on the mice in experiment 2 and 3, but the histopathology showed no significant difference between lymphedema hindlimb and control hindlimb in these experiments. Further studies including histopathology are needed for this model to clarify whether lymphedema is induced on a histological level. Experiments 2 and 3 have not yet been published and we therefore cannot refer to them.

While using µCT scans to measure hindlimb volume can be argued to be more objective than using the water displacement method or circumferential measurements, it still has its limitations. The measuring technique is expensive, time-consuming and requires access to a µCT-scanner and analyzing software.

One of the biggest challenges with rodent lymphedema models in general, have been spontaneous lymphedema resolution, unless excessive radiation was performed[25]. When developing this model, we tested several different doses of radiation to find a dose that would induce lasting lymphedema without causing severe morbidity[26]. Previously, lymphedema models have not been standardized in the methods of lymphedema induction or outcome assessments. Oashi et al.[20] used a single dose of 30 Gy irradiation, and ligated each lymphatic vessel at three separate points. In that study, the surgical procedure took 90 min to perform. Although the method presented in this article can be considered time-consuming, the surgical part of the procedure can still be performed approximately twice as fast as the method presented by Oashi et al.[20]. They also had a follow-up period of 6 months, which is considerably longer than any of the studies presented in this article. However, they only included one mouse and they manually measured limb circumference to assess the swelling, whereas the volumes presented in this article was measured on 31 mice using µCT scans and 3D analysis software. Komatsu et al.[30] removed the inguinal lymph nodes and the associated peripheral lymph vessels and fat tissue using an electric knife. Using an electric knife might be a simpler approach which does not require microsurgical training, but the induced edema resolved after day 4 while the method presented in this article offers consistent lymphedema lasting at least 8 weeks.

This protocol will hopefully enable researchers to consider the limitations and advantages of the revised lymphedema model. The protocol should also assist researchers to successfully replicate the model. The method can be used in future observational and interventional studies to understand the pathophysiology of lymphedema and research novel treatment options. In future studies, it would also be interesting to have a follow-up longer than 8 weeks to observe just how long the induced lymphedema lasts. It would also be interesting to observe the effect of performing more targeted irradiation of the mice pre- and post-surgery. This could be done by performing a CT scan and planning a target volume. In future studies, this model could also be supported by fluorescence-guided lymphatic imaging, perometry or bioimpedance studies. This method offers statistically significant lymphedema lasting at least 8 weeks, which has been measured directly via CT volumetric in three separate experiments by different lead investigators.

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DISCLOSURES:

The authors have nothing to disclose.

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FIGURE 1

FIGURE 2

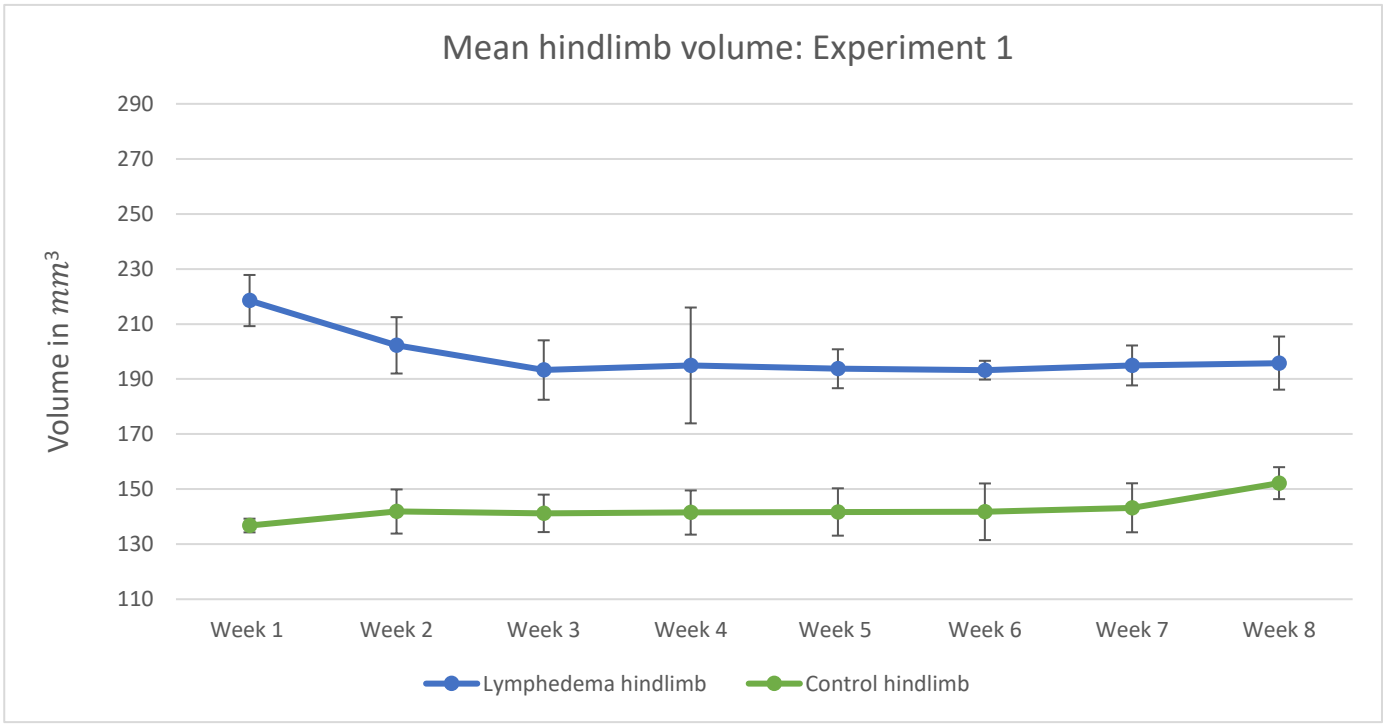

FIGURE 3

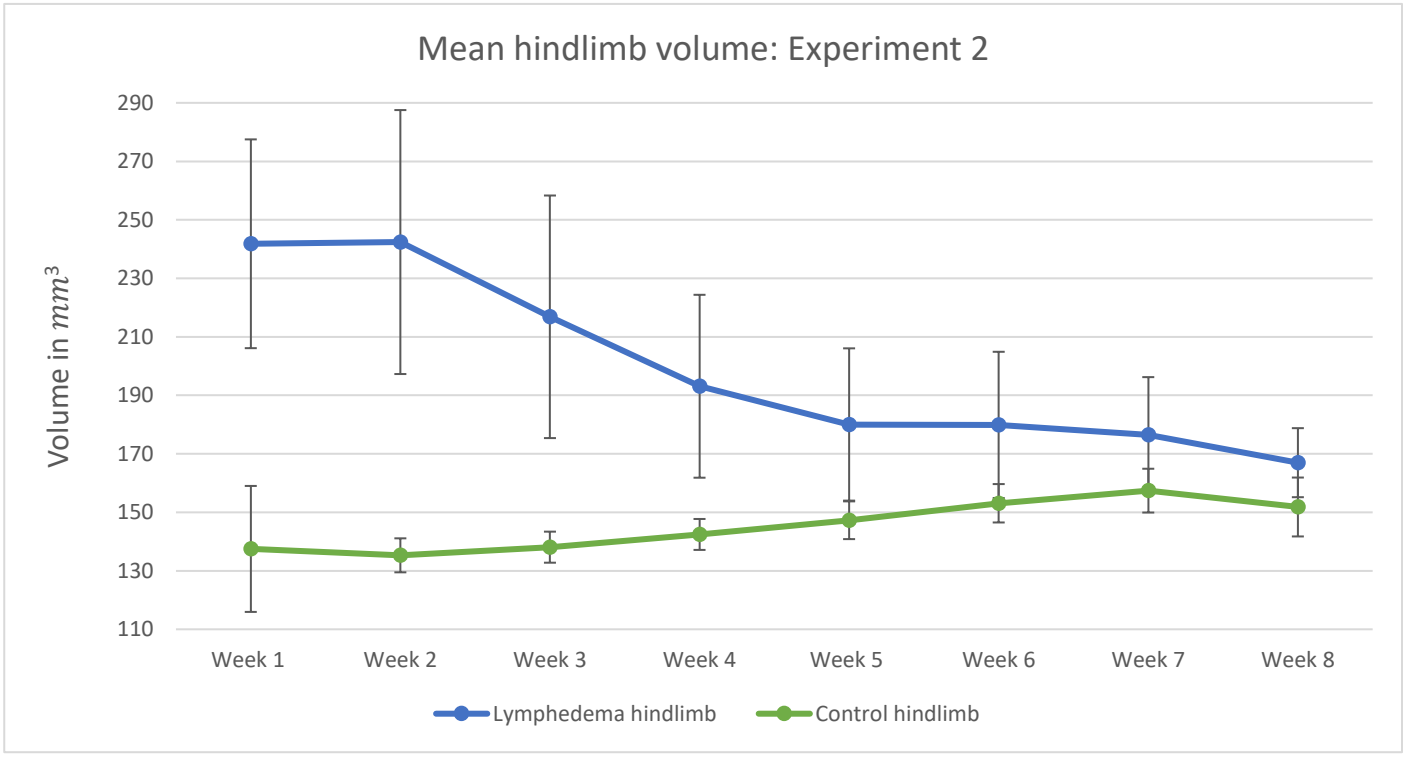

FIGURE 4

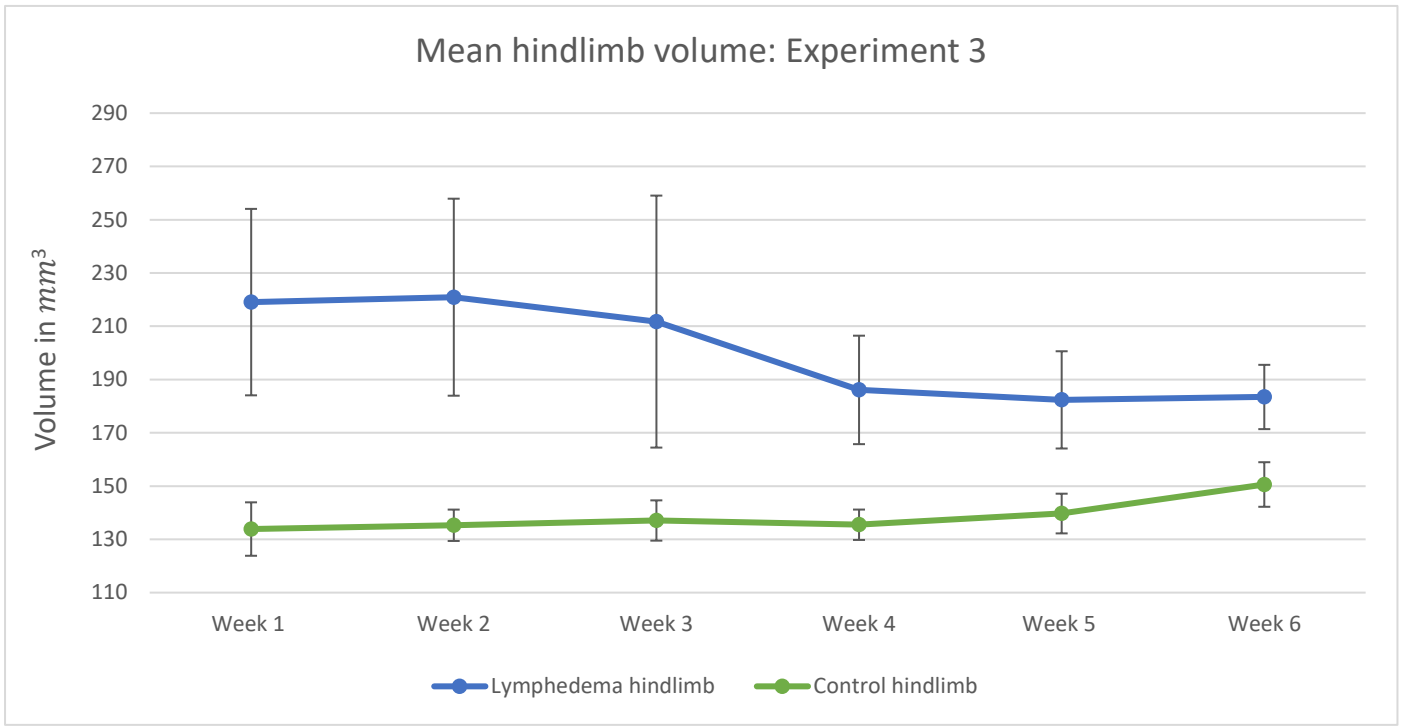

FIGURE 5

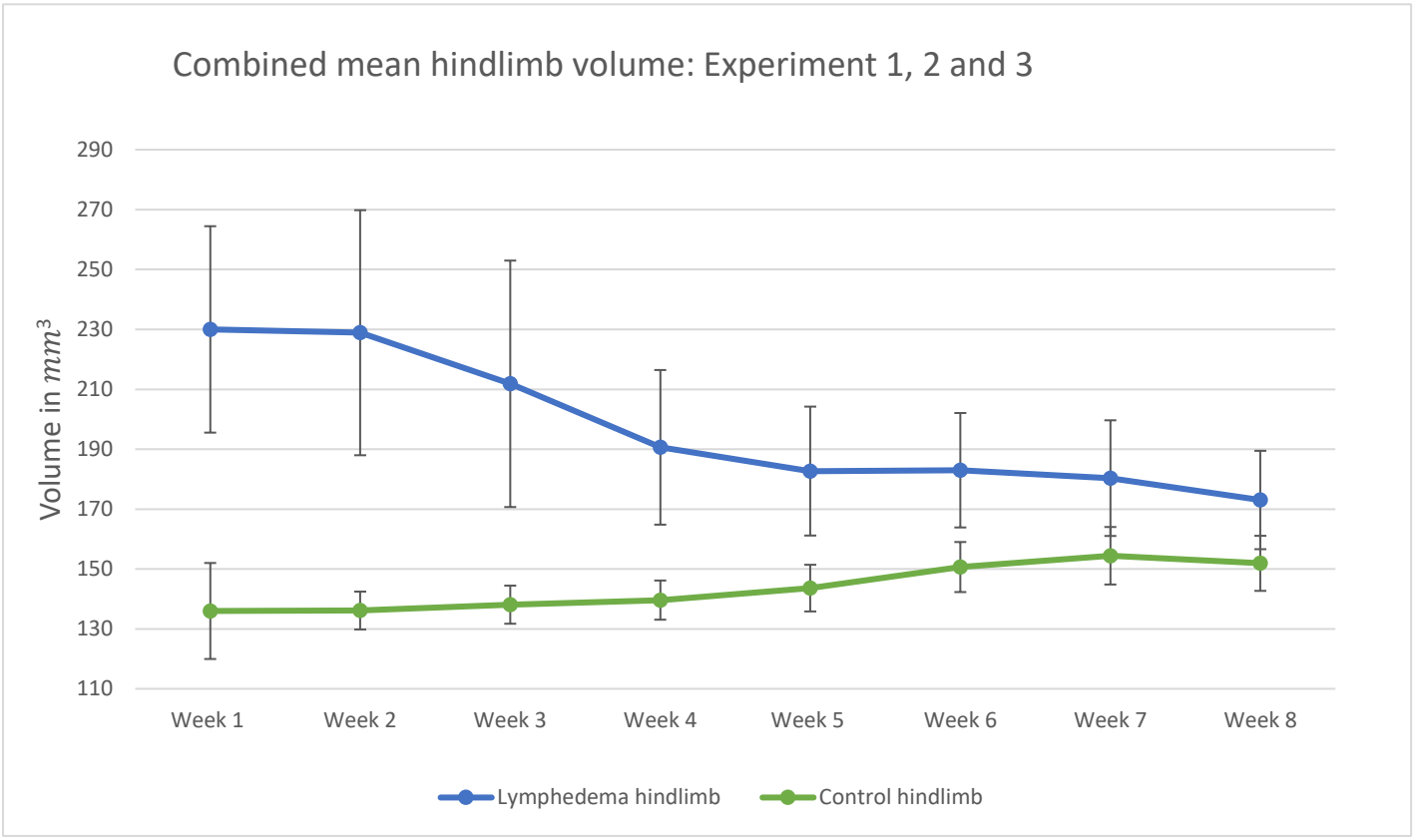

Table1

Week	Lymphedema volume in mm^3 (n = 4)	Control volume in mm^3 (n = 4)	P-value	95% Confidence interval
1	218.53 ± 9.3	136.78 ± 2.48	0.002	53.77–109.73
2	202.25 ± 10.24	141.88 ± 8.02	0.066	(-6.53)–127.28
3	193.28 ± 10.80	141.20 ± 6.80	0.060	(-3.7)–107.85
4	194.95 ± 21.05	141.50 ± 8.03	0.224	(-41.85)–148.75
5	193.75 ± 7.07	141.70 ± 8.60	0.051	(-0.27)–104.37
6	193.23 ± 3.42	141.78 ± 10.29	0.054	(-1.56)–104.46
7	194.95 ± 7.26	143.23 ± 8.90	0.050	0.17–103.28
8	195.8 ± 9.65	152.18 ± 5.81	0.009	19.88–67.38

Table2

	Experiment 1		Experiment 2	
Week	Absolute difference (mm^3)	Relative difference (%)	Absolute difference (mm^3)	Relative difference (%)
1	81.75 ± 7.20	0.60 ± 0.04	104.34 ± 25.96	0.76 ± 0.23
2	60.38 ± 17.21	0.43 ± 0.14	107.12 ± 44.33	0.79 ± 0.33
3	52.08 ± 14.35	0.37 ± 0.11	78.77 ± 39.45	0.57 ± 0.28
4	53.45 ± 24.51	0.38 ± 0.19	50.67 ± 29.94	0.36 ± 0.21
5	52.05 ± 13.46	0.37 ± 0.11	32.74 ± 24.66	0.22 ± 0.17
6	51.45 ± 13.63	0.36 ± 0.11	26.80 ± 22.35	0.18 ± 0.14
7	51.73 ± 13.26	0.36 ± 0.11	19.04 ± 17.22	0.12 ± 0.11
8	43.63 ± 6.11	0.29 ± 0.04	15.15 ± 11.70	0.10 ± 0.08

Experiment 3		Experiment 1, 2 and 3 combined	
Absolute difference (mm^3)	Relative difference (%)	Absolute difference (mm^3)	Relative difference (%)
85.20 ± 35.05	0.64 ± 0.27	94.02 ± 29.57	0.69 ± 0.24
85.63 ± 37.94	0.63 ± 0.29	92.77 ± 41.68	0.68 ± 0.31
74.67 ± 49.57	0.54 ± 0.38	73.74 ± 41.51	0.53 ± 0.31
50.62 ± 16.35	0.37 ± 0.11	51.01 ± 24.03	0.37 ± 0.17
42.67 ± 11.81	0.31 ± 0.07	39.08 ± 20.02	0.27 ± 0.14
32.86 ± 10.90	0.22 ± 0.08	32.32 ± 18.96	0.21 ± 0.13
-	-	25.92 ± 21.15	0.17 ± 0.15
-	-	21.15 ± 15.96	0.14 ± 0.10

Table3

Week	Lymphedema volume in mm^3 (n = 15)	Control volume in mm^3 (n = 15)	P-value	95% Confidence interval
1	241.82 ± 35.69	137.48 ± 21.54	<0.001	82.21–126.47
2	242.41 ± 45.13	135.29 ± 5.81	<0.001	69.33–144.89
3	216.85 ± 41.47	138.08 ± 5.31	<0.001	45.15–112.39
4	193.10 ± 31.27	142.43 ± 5.29	<0.001	25.15–76.18
5	180.03 ± 26.03	147.29 ± 6.45	0.002	11.72–53.76
6	179.89 ± 25.00	153.09 ± 6.56	0.004	7.74–45.85
7	176.45 ± 19.77	157.41 ± 7.49	0.008	4.35–33.71
8	166.97 ± 11.8	151.82 ± 10.07	0.002	5.18–25.12

Table4

Week	Lymphedema volume in mm^3 (n = 12)	Control volume in mm^3 (n = 12)	P-value	95% Confidence interval
1	219.06 ± 35.00	133.86 ± 10.02	<0.001	51.66–118.74
2	220.90 ± 36.98	135.27 ± 5.89	<0.001	49.33–121.94
3	211.74 ± 47.30	137.07 ± 7.56	0.002	27.24–122.11
4	186.09 ± 20.36	135.47 ± 5.70	<0.001	34.98–66.27
5	182.35 ± 18.25	139.68 ± 7.45	<0.001	31.37–53.98
6	183.44 ± 12.11	150.58 ± 8.37	<0.001	22.42–43.29

Week	Lymphedema volume in mm^3 (Week 1−6 n = 31) (Week 7−8 n = 19)	Control volume in mm^3 (Week 1−6 n = 31) (Week 7−8 n = 19)
1	230.00 ± 34.46	135.99 ± 16.03
2	228.90 ± 40.91	136.13 ± 6.32
3	211.83 ± 41.15	138.09 ± 6.36
4	190.63 ± 25.81	139.62 ± 6.54
5	182.70 ± 21.52	143.62 ± 7.79
6	182.98 ± 19.11	150.66 ± 8.36
7	180.34 ± 19.31	154.43 ± 9.60
8	173.04 ± 16.42	151.89 ± 9.19

P-value	95% Confidence interval
<0.001	78.19−109.84
<0.001	70.47−115.07
<0.001	51.53−95.95
<0.001	38.15−63.87
<0.001	28.36−49.79
<0.001	22.18−42.47
<0.001	11.61−40.22
<0.001	10.35−31.94

Table of Materials

Click here to access/download;Table of Materials;60578_Materials.xls

Name of Material/Equipment	Company	Catalog Number	Comments/Description
10-0 Nylon suture	S&T	12051-10	
6-0 Nylon suture - Dafilon	B Braun	C0933112	
Coagulator - ICC 50	ERBE		
Cotton tipped applicators	Yibon medical co		
Dissecting forceps	Lawton	09-0190	
Elastic retractors	Odense University Hospital		
Electrical clipper	Aesculap	GT420	
Fentanyl 0,315 mg/ml	Matrix		
Heating pad - PhysioSuite	Kent Scientific Corp.		
Isoflurane 1000mg Attane	Scan Vet		
Isoflurane vaporizer - PPV	Penlon		
Micro jewler forceps	Lawton	1405-05	
Micro Needle holder	Lawton	25679-14	
Micro scissors	Lawton	10128-15	
Micro tying forceps	Lawton	43953-10	
Microfine U-40 syringe 0,5ml	BD	328821	
Microlance syringe 25g	BD		
Microlance syringe 27g	BD		
Midazolam 5 mg/ml (hameln)	Matrix		
Needle holder - Circle wood	Lawton	08-0065	
Non woven swabs	Selefa		
Opmi pico microscope F170	Zeiss		
Patent blue V - 25 mg/ml	Guerbet		
Scissors - Joseph	BD	RH1630	
Siemens INVEON multimodality pre-clinical scanner	Siemens pre-clinical solutions		
Source of radiation - D3100	Gulmay		
Stata Statistical Software: Release 15	StataCorp LLC		
Temgesic - 0,2 mg	Indivior		
Vet eye ointment - viscotears	Bausch & Lomb		

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Author(s): Alexander Wiinholt, Mads Gustaf Jørgensen, Amar Bučan, Farina Dabei, Jens Ahm Sørensen

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Dear Dr Cao

Thank you for the opportunity to revise the manuscript and video "A revised method for inducing secondary lymphedema in the hindlimb of mice." The suggestions offered by the reviewers and editors have been very helpful, and we appreciate the insightful comments.

Editorial and production comments:
Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have thoroughly proofread the manuscript for spelling and grammar issues.

2. 1.1.1: Please specify the age, gender and strain of mouse.

Age, gender and strain of mouse has been written at 1.1.1. line 121-122

3. 2.2.1: What is used here to draw different solutions?

Needle and syringe are added to 2.2.1 and 2.3.1 line 160-162 and 169-170.

4. 2.2.2: How to mix, by pipetting?

The mixing process has been described at 2.2.2 and 2.3.2. Line 164-165 and 169-170

5. 6.1: What is done after post-surgery irradiation? Are any measurements done?

No measurements are done immediately after post-surgery irradiation. As figure 1 depicts the first microCT-scan happens 4 days after post-surgery irradiation. The mice are scanned every week, but the measurements made on the scans in INVEON research software can be done whenever the researcher wants.

6. In the protocol, please add how to obtain the data presented in representative results section. These steps do not have to be included in the video.

We are glad that the measuring is of interest and we would have liked to include it. However, writing the measuring process in protocol format (step by step) would result in the protocol exceeding the 10 page maximum limit. Our department plan on writing a separate manuscript for the measuring process including the process of microCT scanning the mice. If that manuscript would prove to be of interest, we would like the video to be produced by JoVE.

7. Please number the figures/tables in the sequence in which you refer to them in the manuscript text.

This has been done for all figures and tables.

Changes to be made by the author(s) regarding the video:
1. Please update the video according to the revised manuscript.

The video has been updated.

2. 01:45-01:59: Please do not show anesthesia step in the video.

The step has been removed

3. 04:05-04:45, 05:45-06:00: Such details in the video are not included in the manuscript. Please cross-reference the video narration with the protocol text.

04:05 is step 4.5.1 in the manuscript we have tried to elaborate this step. Line 268-269.

04.22-04.45 is 4.6, 4.6.1 and 4.6.2 in the manuscript. We have tried to elaborate by including the abbreviations used in the video. Line 278-285.

05:45-06:00 is step 4.10, 4.10.1, 4.10.2 and 4.10.3 in the manuscript we have tried to cross reference the video. Line 316-324.

4. The music competes with the human voice at times. We recommend lowering the audio level of the music by 3-6 dB.

The audio level of the music has been lowered.

5. Please upload a revised high-resolution video here:
https://www.dropbox.com/request/55mSzIonH4orAVoj2SeI?oref=e

Vet review:

Thank you for your comments and review.

1. 1:03 and lines 114-115. *Improvement requires* Gas anesthesia is described, but it not used in the video. Clarify to state that isoflurane or injectable anesthetics can be used. Change in text sufficient: Yes

   The gas anesthesia was used during the filming of the video but is not shown due to the rule about not showing anesthesia in JoVE videos. We have included a statement about isoflurane or injectable anesthetics page line 124-125. And tried to clarify the procedure in line 131-133.

2. Line 156. *Not acceptable* What is the concentration of the buprenorphine? Provide the concentration of the buprenorphine. Change in text sufficient: Yes

Added to line 169.

3. 3:00. *Not acceptable* Skin preparation is not obvious in the video. Include a description of the aseptic preparation of the skin using alcohol/chlorhexidine or alcohol/povidone iodine. Change in text sufficient: Yes

   Added to line 227.

4. Line 211+. *Not acceptable* There needs to be a statement that sterile surgical instruments and gloves are used. Include a statement that sterile surgical instruments and gloves are used. Change in text sufficient: Yes

   Added to line 153-154.

5. 3:25 and line 144. *Not acceptable* The open surgical wound should not come in contact with a non-sterile surface. In line 144 it state that the operative surface is not sterile. State that the operative surface is sterile. Change in text sufficient: Yes

   Added to line 156.

6. 4:21 to 5:00. *Not acceptable* Fur overlaps the surgical area and appears to be in contact with the instruments. This needs to be re-done with a larger area of fur removed so it doesn't overlap the surgical site or touch the instruments. This can cause wound contamination and infection. For this procedure plucking the fur from the incision site or using a depilatory cream may be better than clippers. Change in video required: Yes
7. 4:21 to 5:30. *Not acceptable.* There appears to be clipped bits of fur in the surgical site. This can cause wound contamination and infection. Change in video required: Yes

6 and 7. We fully acknowledge that the fur is overlapping the surgical area and is in contact with part of the instruments. However, the proximal lymph vessel lies so close to the trunk of the mouse, that to ensure no hair is visible and overlapping the surgical area, we would have to remove fur from the back and tail base of the mouse as well. Even if we were to remove hair from such a large area, microscopic hair would still be visible through the microscope. In the JoVE article "Cavernous Nerve Stimulation and Recording of Intracavernous Pressure in a Rat" at 1:59 small hairs can also be seen in the surgical area and being in contact with cottonswaps and instruments. We agree that wound contamination and infection should be avoided at all times, but the procedure requires the mouse to get moved and placed in different positions to access the different lymph nodes and vessels. It is virtually impossible not to get microscopic hairs into the surgical area during the procedure which will show in the video at the parts recorded with maximum magnification. We have emphasized that it is important to thoroughly rinse the leg with sterile saline before closing the incision at line 328-330. Should these steps be redone in the video we would need longer than 2 weeks to order new mice, have them acclimatized, borrow camera equipment, plan a suitable date for operation and edit it into the video.

8. 6:32 and onwards, lines 307-308. *Not acceptable* A good scientific justification for not closing the surgical site needs to provided. In the text, provide a scientific justification for leaving a gap between the skin edges. References are needed. Change in text sufficient: Yes

   Yes, we fully agree, thank you for letting us know. We have included scientific justification and reference at line 525-527.

9. Lines 319+. *Not acceptable* There is no description of the post-operative care of the open wound. Describe any post-operative care of the wound. At a minimum it should have antibiotic ointment to protect the exposed tissue. Change in text sufficient: Yes

   Antibiotic ointment was not used unless there were signs of infection. The veterinarians at the local animal facility supervised the animals and made sure that there were no signs of infection. We have included a statement at line 353-354.

Reviewers' comments:
Reviewer #1: Manuscript Summary:

This manuscript presents a combined irradiation + surgical method to produce lymphedema in mice. There are a few items that the authors should clarify to help others successfully reproduce their model.

Thank you for your review and comments.

Major Concerns:

None

Minor Concerns:

1. Has this procedure been performed in multiple strains of mice? There might be strain-specific advantages or disadvantages worth noting.

No, it has only been tested on C57BL6 mice. We agree that it should be included and have now included this at line 121-122.

2. Does the procedure work effectively on both male and female mice?

We have only tested it on female mice, we included this at line 121-122.

3. Is there a preferred age for the mice at which the procedure should be performed?

In our experiments the mice where 9-weeks old, this have now been included at line 121-122. Questions 1-3 was all asked by the editor as well, and it was requested to be included at step 1.1.1 in the protocol.

4. Line 196 - Please clarify that the nosecone is only for gas delivery and not anesthesia. This wasn't entirely clear especially because the previous procedure uses isoflurane anesthesia. Also, is 100% oxygen used, or medical gas mixture?

We have tried to clarify this at line 211-212.

5. Line 214 - Is any fur removed or germicidal scrub applied prior to making the incision?

Yes, the fur was shaved using electrical clippers this has been written at line: 202- 203. We have included a statement about germicidal scrub at line 227.

6. Line 484 - Is the intended purpose of this sentence to say to finish as quickly as possible in order to avoid an excessively long surgical period?

Yes exactly, we should have clarified that. It is also to prevent the injectable anesthesia from losing effect during the procedure. We have tried to elaborate at line 514-517.

7. Figure legends - please clarify that the number of subjects is per group. It might be confused that this was the total number for each experiment.

That is a good point. We have tried to clarify this in the figure legends. Figure 2,3 and 4.

Reviewer #2: Manuscript Summary:
This article describes the protocol for inducing lymphedema in the hindlimb of mice. The described pre-clinical model last at least 8 weeks. It is obtained by the use of pre- and post-surgical radiotherapy and by the lymphadenectomy of the inguinal and popliteal lymph nodes as well as the ligature of several lymphatics.

Thank you for your review and comments.

Minor Concerns:
Spontaneous lymphedema resolution is the main common limitation in many published animal models. The rodent limb appears to be an interesting and eligible animal model, mainly for experimental reconstruction of the lymphatic function, because it is easily accessible for vascularized lymph node transfers and for lymphatico-venous anastomoses. The content of the article and the relevance of the work are of interest, however, there are some minor indications that can help to improve the article:

1. The limitations of this model as well as the lymphedema evaluation technique used (volumetry) should be described in greater depth in this article. The use of µCT scans is more objective than

other volumetric measurements such as the water displacement method or the circumferential measurements. However, this method also has limitations, please explain them.

We have tried to elaborate on the limitations of the article at line 534-544.

2. Indicate in the discussion section that other parameters have to be studied and correlated in this model in further studies. Fluorescence-guided lymphatic imaging, perometry or bioimpedence studies supporting this protocol would have given the article a greater scientific relevance.

We agree that this is relevant. We have included this at line: 572-573.

3. It is indicated in the article that this procedure has been previously used in three separate experiments, if these have been already published, please introduce the bibliographic references in the article.

Experiment 1 has been published and is referenced at line 384. We would have liked to be able to reference to Experiment 2 and 3 but they have not been published yet we have included this in the discussion line 539-541.

Reviewer #3: Manuscript Summary: Thanks for sharing an animal model method to study secondary lymphedema, and discussing advantages and limitations.

Thank you for your review and comments.

Major Concerns:

Minor Concerns:

1- the title suggests a "revised" method. In the introduction section, could you please explain what is being revised? Simply highlight the main differences between the original model and the revised one?

Yes, we have tried to explain at line 96-98.

2- Line#54: I suggest rephrasing to "that leads to localized tissue swelling", not "edema" - because edema has a different pathophysiology.

Thank you, we have rephrased it at line: 54-55.

Reviewer #4: Manuscript Summary:
The authors describe the induction of hindlimb lymphedema in mice by means of 2 irradiation doses and surgery. Even though this description may be helpful for lymphatic researchers (especially those who start with the mouse model), a few refinements of the text and protocoll should be done. From a methodoligcal point of view, the authors have used a well-established

mouse model with (minor) modification and used established volumetric techniques. It is a pitty, they did not perform histology and lymphatic imaging.

Thank you for your review and comments.

Major Concerns:
1. The authors often mention "significant lymphedema". I think this should be debated: Even though the authors induce LE by means of surgery and popliteal/inguinal lymphadenectomy, they unfortunately missed the opportunity to analyze the limbs histopathologically. There are very few studies reporting true lymphedema in the rodent hindlimb (for instance pubmed ID 28678084) and it is important to inform the reader that without histology, they are basically just analyzing "edema". Hence, histopathological analyses would be nice to support the assumption that this edema is true chronic LE, ie the tissue undergoes the typical changes with fibrofatty and immune cell infiltration and lymphatic vessel abnormalities. I understand that this is much more work but without histology, it is not legitimate to talk about LE. If the paper should be accepted w/o histology, this should be discussed and acknowleged appropriately. Please also include a section on lymphatic imaging, which is another important missing part.

We agree that histopathology is important to support the fact that we have induced true chronic lymphedema. We fully acknowledge that this is needed in the discussion. We would like to refer to experiment 1 "Quantification of Chronic Lymphedema in a Revised Mouse Model" by Jørgensen et. al. which includes histopathology. This will help clarify that some of the mice included as representative results had induced lymphedema. Experiment 2 and 3 showed no significant difference between lymphedema and control hindlimb in the histopathology. This will also be stated in the discussion. This has been discussed in line 534-544.

2. Introdoction, line 86/86: This is not accurately referenced. The mouse tail model is very reliable but of course more difficult to understand from a translational perspective. In contrast, the limb models are much more unrealiable and do often only provide "acute" surgical edema, which is misinterpreted as LE. Many lymphatic researcher however start with the mouse or rat hindlimb and hope to produce chronic LE with minimally invasive procedures (which never works). Therefore, I welcome the present protocol but the above-mentioned limitations are important and should be acknowledged.

The reference in question from the manuscript is:
"But research has shown, that the tail model may result in fluctuations in duration and robustness of the developed lymphedema, whereas a combination of radiation and surgery in the hindlimb of rodents induce a more stable lymphedema"

It was written according to this statement from the discussion in "Animal models in surgical lymphedema research a systematic review "by Frueh et al:

“After the introduction of the rat hind limb as an easily accessible, cost-effective, and reliable lymphedema model [32,33], the use of rodents has gained great popularity. The combination of radiation and surgery has proven to induce most effectively a stable lymphedema in both rats and mice [34,38,43]. The rodent tail model represents another lymphedema model, reported by Slavin et al. [39] Compared to the hind limb model, anatomy and surgical techniques are simple and well reproducible. It has been used for research of surgical lymphedema treatment, gene therapy, and molecular aspects of lymphangiogenesis [39,51,53,54]. However, the exact surgical technique may significantly vary, resulting in fluctuations in duration and robustness of the developed lymphedema.”

We agree that this could have been more accurately referenced and have tried to correct it at line 85-90. We also agree that the mouse tail model is reliable and we realize that we should have stated more clearly that the “fluctuations in duration and robustness of the developed lymphedema“ happens because the “the exact surgical technique may significantly vary”. We agree that many lymphatic researchers start with the mouse or rat hindlimb and try to produce chronic LE with minimally invasive procedures and that this fails. That is why we planned to make this article, presenting a hindlimb model we believe to be reliable.

3. Have the authors considered to apply the radiation in a more targeted way, for instance by performing a CT scan with planning the target volume? Importantly, in my experience, I am still not sure that radiation is critical for inducing LE in the hindlimb (especially w/o histology PROOFING that this is really LE).

Unfortunately we did not have the means to apply radiation in a more targeted way at our research facility but it is certainly relevant. We have included it in the discussion: line 574-576. In regard to whether radiation is critical, we refer to “Quantification of Chronic Lymphedema in a Revised Mouse Model” by Jørgensen et. al. In this article it can be seen that the surgical procedure alone did not result in edema or histopathological changes.

4. Finally, i do not understand what is the difference between the 3 "experiments"? If I understand correctly, three different investigators have performed them. The data of the first one is already published so I am a bit concerned about originality. Why not just combine the data of experiments 2 and 3 and report 6-week follow-up results? Would be much more easier to read with less redudancy (too many figures and tables, one of each is enough).

That is correct, they are all co-authors of the article. The data of the first experiment is published, we only use part of the results and it is all with the consent of the main author. We would like to avoid combining data from experiment 2 and 3 and report a 6-week follow-up as a result. We think that it is important to show that the model maintains lymphedema for at least 8 weeks, and it would be regrettable to discard the results of the 15 mice in experiment 2 (week 7 and 8.) That would leave us with only 4 mice in week 7 and 8, which would weaken the

statement that the model induces lymphedema for at least 8 weeks. Therefore, we have presented all 3 experiments separately to clearly highlight that we have data from 19 mice with 8-week follow-up. And 12 mice with 6-week follow up. We think this is important, to ensure that the data does not get misinterpreted. To highlight a limitation of the method we also think it is important to illustrate that Experiment 2, did not yield as stable a lymphedema as experiment 1 and that the lymphedema volume seems to decrease even further in week 7-8 (experiment 2). We think this need to be depicted to give readers a precise representation of the different outcomes of using this model.

Minor Concerns:

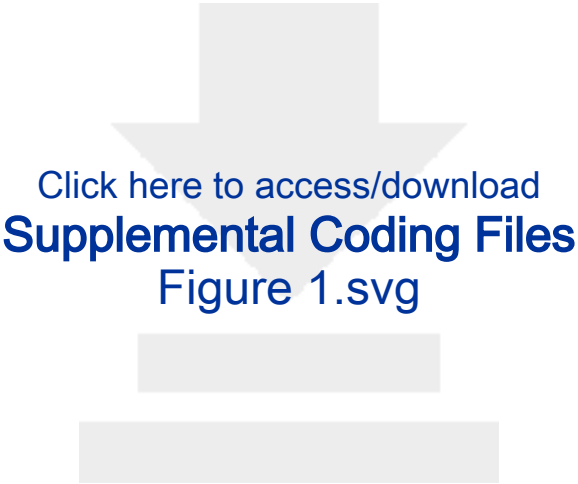
1. I do not agree on microsurgical training opportunity: It may be an opportunity to work with a microscope. However, because the model does not contain microsurgical anastomoses, it is not really challenging. Any medical student can learn this procedure within a few hours (this is at least our experience).

Suturing with a 10-0 suture requires knowledge in handling microsurgical instruments for this procedure to be performed effectively and without the risk of puncturing the lymph vessel with the needle. We think it is important that researchers new to the procedure train and acquire a basic knowledge of how to handle microsurgical instruments and suture with them. In our experience the procedure required practice, and we therefore think it is important to state this.

2. Patent Blue injection: I prefer to inject it into the dermis of the toes, this yields more pressure with better uptake into the lymphatic system. It is absolutely necessary to provide the user with the size of the canule for injection (it does not work with commonly sized canules, the have to be very small). Gauge?

We agree that the gauge should be provided. It has been added to line 267.

Figure 1 in vector format (SVG)



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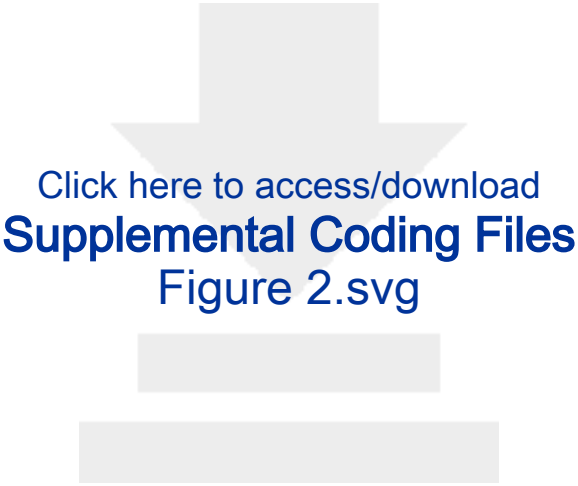
Supplemental Coding Files

Figure 1.svg

Figure 2 in vector format (SVG)



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Supplemental Coding Files

Figure 2.svg

Figure 3 in vector format (SVG)

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Supplemental Coding Files

Figure 3.svg

Figure 4 in vector format (SVG)

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Figure 4.svg

Figure 5 in vector format (SVG)

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Figure 5.svg